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PAPER

Proteomic analysis of ovarian cancer cell responses to cytotoxic gold compounds†

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Platinum-based chemotherapy is the primary treatment for human ovarian cancer. Overcoming platinum resistance has become a critical issue in the current chemotherapeutic strategies of ovarian cancer as drug resistance is the main reason for treatment failure. Cytotoxic gold compounds hold great promise to reach this goal; however, their modes of action are still largely unknown. To shed light on the underlying molecular mechanisms, we performed 2-DE and MS analysis to identify differential protein expression in a cisplatin-resistant human ovarian cancer cell line (A2780/R) following treatment with two representative gold compounds, namely Auranofin and Auoxo6. It is shown that Auranofin mainly acts by altering the expression of Proteasome proteins while Auoxo6 mostly modifies proteins related to mRNA splicing, trafficking and stability. We also found that Thioredoxin-like protein 1 expression is greatly reduced after treatment with both gold compounds. These results are highly indicative of the likely sites of action of the two tested gold drugs and of the affected cellular functions. The implications of the obtained results are thoroughly discussed in the frame of current knowledge on cytotoxic gold agents.

Introduction

Ovarian cancer is the second among gynecological cancers in number of new cases and the first among gynecological cancers in rate of deaths in Western countries.[1,2]

Platinum-based chemotherapy, such as cisplatin, is the primary treatment for human ovarian cancer. The occurrence of intrinsic or acquired tumor chemoresistance remains the major determinant of chemotherapy failure and an unfavorable clinical outcome.[3,4] Several mechanisms have been implicated in the development of tumor drug resistance.[4–7]

Owing to the great clinical success of cisplatin, several new platinum and non-platinum metal compounds have been prepared, characterized and evaluated pharmacologically as anticancer agents.[8] In recent years, research has increasingly focused on cytotoxic gold compounds as drug candidates.[9] Gold(III) complexes display the same electronic configuration (d^8) and similar structural and reactivity features of platinum(II) complexes[10] but the respective mechanisms appear to be radically different. Remarkably, a number of gold(III) complexes have been found to possess promising pharmacological profiles *in vitro* and some of them also *in vivo*.[11,12]

Previous studies have shown that cytotoxic gold(III) compounds are able to induce cell death through apoptosis,[13,14] essentially triggered by a direct mitochondrial damage.[15,16]

Proteomic profiling offers an excellent opportunity for the identification of proteins that mediate apoptotic pathways when cells are treated with cytotoxic agents.[17] The molecular mechanisms of anticancer metallodrugs are usually very complicated and varied owing to the high reactivity of these compounds toward biomolecules, to their being (in most cases) prodrugs (thus undergoing large chemical transformations and extensive speciation within the biological milieu) and to the large differences in the electronic structure and reactivity existing among the various metal centers. Yim *et al.* examined protein expression in cisplatin-treated HeLa cervical carcinoma cells and found 21 altered proteins.[18]

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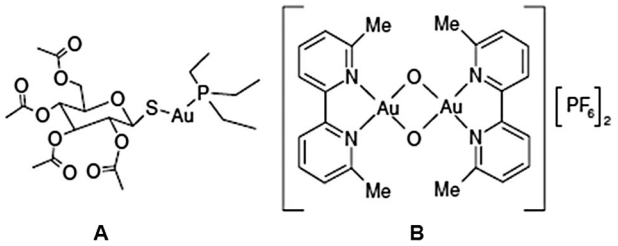

Fig. 1 *Auranofin* (A) and *Auoxo6* (B).

These authors showed that cisplatin induced a marked down-regulation of nuclear factor κB. Yao *et al.* carried out comparative proteomic studies of colon cancer cells in response to oxaliplatin treatment and highlighted a number of proteins that were simultaneously altered in three distinct colon cancer cell lines.[19] These proteins were identified and found to take part in many cellular processes, such as apoptosis, signal transduction, transcription and translation, cell structural organization, and metabolism. Che *et al.* used 2D electrophoresis based proteomic technology to investigate the protein expression profiles of human nasopharyngeal carcinoma SUNE1 cells upon treatment with gold(III) porphyrin 1a.[20] Relevant changes in the expression of a number of proteins engaged in redox metabolism and in the mitochondrial functions were detected suggesting that mitochondria could be a primary target for gold(III) porphyrin 1a.

In our previous work we analyzed proteomic alterations induced by *Auranofin* and *Auoxo6* in a human ovarian cancer cisplatin-sensitive cell line (A2780/S).[21] Six altered proteins were in common between the two treatments. Some of the affected proteins are primarily involved in intracellular redox homeostasis, implying that cell damage is probably the consequence of severe oxidative stress. Here we report the results of a proteomic study on the cellular effects of both gold compounds, *Auranofin* and *Auoxo6* (Fig. 1), on A2780/R (cisplatin-resistant) cancer cells.

From our analysis it emerges that the two different gold compounds cause different proteomic modifications. *Auranofin* mainly acts by altering the expression of Proteasome apparatus proteins, while *Auoxo6* by an expression modification of proteins related to mRNA splicing, trafficking and stability. Furthermore we found that a protein whose expression is reduced after cells treatment with both gold compounds is Thioredoxin-like protein 1, involved in oxidative stress defence.

Experimental procedures

Materials and reagents

Auoxo6 was synthesized as described in ref. 22, and *Auranofin* was obtained from Vinci Biochem. All other chemicals were of analytical grade. RPMI 1640 cell culture medium, fetal calf serum (FCS), and phosphate-buffered saline were obtained from Celbio (Milan, Italy); sulforhodamine B (SRB) was obtained from Sigma (Milan, Italy).

Cell lines

For cytotoxicity studies the cisplatin-sensitive human ovarian carcinoma cell line (A2780/S) and its cisplatin-resistant cell subline (A2780/R) were used. For proteomic studies the A2780/R subline was used. Cell lines were maintained in RPMI1640 medium supplemented with 10% of FCS and antibiotics at 37 °C in a 5% CO_2 atmosphere and subcultured twice weekly.

Cell growth inhibition studies

The cytotoxic effects of *Auranofin* and *Auoxo6* were evaluated against the A2780/S and the A2780/R cell lines according to the procedure described by Skehan.[23] *Auoxo6* was diluted in DMSO and *Auranofin* in ethanol as stock solutions (10 mM). Exponentially growing cells were seeded in 96-well microplates at a density of 5×10^3 cell per well. After cell inoculation, the microtitre plates were incubated under standard culture conditions (37 °C, 5% CO_2, 95% air and 100% relative humidity) for 24 h prior to the addition of study compounds. After 24 h, the medium was removed and replaced with fresh medium containing drug concentrations ranging from 0.003 to 100 μM for a continuous exposure of 72 h for both compounds tested. For comparison purposes the cytotoxic effects of cisplatin, measured under the same experimental conditions, were also determined. Then the cells were fixed with 100 μL of ice-cold 10% trichloroacetic acid (TCA) for 60 min at 4 °C, rinsed 6 times with water and air-dried. Fixed cells were stained with 50 μL of sulforhodamine B (SRB) solution (0.4% SRB/0.1% acetic acid), rinsed with 0.1% acetic acid and air-dried. At the end of the staining period, SRB was dissolved in 150 μL of 10 mM Tris–HCl solution (pH 10.5) for 10 min in a gyratory shaker. Optical density was read in a microplate reader interfaced with the software Microplate Manager/PV version 4.0 (Bio-Rad Laboratories, Milan, Italy) at 540 nm. The IC_{50} drug concentration resulting in a 50% reduction in the net protein content (as measured by SRB staining) in drug-treated cells as compared to untreated control cells was determined after 72 h of drug exposure. The IC_{50} data represent the mean of at least three independent experiments.

Sample preparation and 2D gel electrophoresis

Cells were seeded in tissue-culture plates at 5×10^4 cells mL^{-1} (total volume 30 mL) and incubated overnight, then exposed to concentrations of the study compounds equal to 72 h-exposure IC_{50} values for 24 h. At the end of incubation cells were washed with phosphate-buffered saline, then were scraped in RIPA buffer [50 mM Tris–HCl, pH 7.0, 1% NP-40, 150 mM NaCl, 2 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid, 100 mM NaF] containing a cocktail of protease inhibitors (Sigma). Cells were sonicated (10 s) and protein extracts were clarified by centrifugation at 8000*g* for 10 min. Proteins were precipitated following a chloroform/methanol protocol[24] and pellets were resuspended in 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and 20 mM dithiothreitol (DTT). Three independent experiments were performed and each sample was run in triplicate to assess biological and analytical variations. Isoelectrofocusing (first dimension) was carried out on nonlinear wide-range immobilized pH gradients (IPGs; pH 3.0–10; 18 cm-long IPG strips; GE Healthcare, Uppsala, Sweden) using an EttanTM IPGphor™ system (GE Healthcare, Uppsala, Sweden). Analytical-run IPG strips were rehydrated with 60 μg of total proteins in 350 μL of lysis buffer and 0.2% carrier ampholyte for 1 h at 0 V and for 8 h

at 30 V, at 20 °C. The strips were focused at 20 °C according to the following electrical conditions: 200 V for 1 h, from 300 to 3500 V in 30 min, 3500 V for 3 h, from 3500 to 8000 V in 30 min, and 8000 V until a total of 80 000 V h^{-1} was reached. For preparative gels, 18 cm IPG strips (pH 3–10 NL) were rehydrated overnight for 20 h at room temperature in 350 μL of rehydration buffer containing 8 M urea, 2% w/v CHAPS, 0.5% DTT, and 0.5% IPG buffer with the same pH range as the Immobiline DryStrips and a trace of bromophenol blue. Rehydrated strips were rinsed in double-distilled water to remove urea crystals. Samples (up to 1 mg) were cup-loaded near the anode of the IPG strips using an Ettan IPGphor cup-loading manifold (GE Healthcare) according to the manufacturer's protocol. After focusing, analytical and preparative IPG strips were equilibrated for 12 min in 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 2% DTT in 0.05 M Tris–HCl buffer, pH 6.8, and subsequently for 5 min in the same urea/sodium dodecyl sulfate/Tris–HCl buffer solution where DTT was substituted with 2.5% iodoacetamide. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 10 °C and 40 mA per gel constant current until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silver nitrate as previously described;[25] MS-preparative gels were stained with colloidal Coomassie.[26]

Image analysis and statistics

Gel images were acquired with an Epson expression 1680 PRO scanner. For each condition, three biological replicates were performed and only spots present in all the replicates were taken into consideration for subsequent analysis. Computer-aided 2D image analysis was carried out using ImageMaster 2D Platinum version 6.0 (GE Healthcare). Relative spot volume ($\%V = 100 \times V$ single spot/V all spots, where V = integrated OD over the spot area) was used for quantitative analysis in order to reduce experimental errors. The normalized intensity of spots on three replicates of 2D gel was averaged and standard deviation was calculated for each condition. The mean values were compared among the three different conditions (control cells, *Auranofin*-treated cells and *Auoxo6*-treated cells) by analysis of variance followed by a two-tailed non-paired Student's *t*-test with ORIGIN 7.5 (Microcal Software, Inc.). $P < 0.05$ was considered statistically significant.

Protein identification by mass spectrometry

Protein identification was carried out by peptide mass fingerprinting on an Ettan matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) Pro mass spectrometer (Amersham Biosciences), as previously described.[27] Spots, visualized by a colloidal Coomassie staining protocol, were manually excised, destained and acetonitrile-dehydrated. Successively, they were rehydrated in trypsin solution and in-gel protein digestion was performed by overnight incubation at 37 °C. From each excised spot, 0.75 μL of recovered digested peptides were prepared for MALDI-TOF MS by spotting them onto the MALDI target, allowing them to dry and then mixing them with 0.75 μL of matrix solution [saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid]. After application of the matrix to the dried sample and drying, tryptic peptide masses were acquired. Mass-fingerprinting searching was carried out in the NCBInr and Swiss-Prot databases using Mascot (Matrix Science, London, UK, http://www.matrixscience.com). Protein identification was achieved on the basis of corresponding experimental and theoretical peptide-fingerprinting patterns. A mass tolerance of 100 ppm was allowed and only one missed cleavage site accepted. Alkylation of cysteine by carbamidomethylation was assumed as a fixed modification, whereas oxidation of methionine was considered a possible modification. Criteria used to accept identifications included the extent of sequence coverage, the number of matched peptides and a probabilistic score, as reported in Table 2. Tryptic digests that did not produce MALDI-TOF unambiguous identifications were subsequently acidified with 2 μL of a 1% trifluoroacetic acid solution, and then subjected to electrospray ionization (ESI)–ion trap MS/MS peptide sequencing using an LCQ DECA ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). With the use of ZIP-TIPTM pipette tips for sample preparation (Millipore, Billerica, MA, USA), previously equilibrated in 50% acetonitrile solution and abundantly washed in 0.1% trifluoroacetic acid, acidified samples were enriched. Tryptic peptide elution from the ZIP-TIPTM matrix was achieved with a 70% methanol and 0.5% formic acid solution, and 3 μL of such concentrated sample solutions were then loaded in the nanospray needle. MS/MS database searching was performed by Turbo-SEQUEST (Thermo) and Mascot MS/MS ion search software (www.matrixscience.com) in the Swiss-Prot/TrEMBL or NCBInr databases. Following criteria were applied: MS accuracy: ±1.2 Da, MS/MS mass accuracy: ±0.6 Da, peptide precursor charge: 2+, monoisotopic experimental mass values, trypsin digestion with one allowed missed cleavage, fixed carbamidomethylation of cysteines and variable oxidation of methionine.

Western blotting analysis of proteomic candidates

Cells conditions were the same as those of the 2-D experiments. Samples (30 μg) were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). To confirm the results obtained from 2-D analysis, the relative amount of the Transitional endoplasmic reticulum ATPase (TERA) (spot 17) and the Hypoxia up-regulated protein 1 (HYOU1) (spot 7) was assessed by Western blot with appropriate monoclonal antibodies (Santa Cruz Biotechnology). According to datasheets both antibodies were employed with a 1 : 1000 dilution in 2% milk. For quantification, blots were stained with Coomassie brilliant blue R-250 and subjected to densitometric analysis performed using Quantity One Software (Bio-Rad). Statistical analysis of the data was performed by Student's *t*-test; *p*-values <0.05 were considered statistically significant. The intensity of the immunostained bands was normalized with the total protein intensities measured by Coomassie brilliant blue R-250 from the same blot.

Results

Cell growth inhibition was measured, according to the procedure of Skehan,[23] after 72 h exposure to the compounds. As expected on the basis of our previous results,[11,21] both compounds showed relevant cytotoxic activity, with IC_{50} values typically falling in

Table 1 *In vitro* antiproliferative activity of *Auranofin* and *Auoxo6* against A2780 ovarian carcinoma human cell lines, either sensitive (A2780/S) or resistant (A2780/R) to cisplatin

	A2780/S, IC_{50} (μM) ± DS	A2780/R, IC_{50} (μM) ± DS
Auoxo6		
Mean	2.4 ± 0.5	6.0 ± 0.5
R		2.5
Auranofin		
Mean	0.50 ± 0.10	0.47 ± 0.06
R		0.94
Cisplatin		
Mean	1.5±0.4	26.1±0.1
R		17.4

The experiments were performed in triplicate; *r*, cross-resistance ratios; DS, standard deviation.

the low μM range (Table 1). In addition, both study compounds turned out to be more active than cisplatin against both the cisplatin-sensitive cell line and the resistant one with cross-resistance ratios (*r*) markedly lower (*i.e.* 0.94 and 2.5 for *Auranofin* and *Auoxo6*, respectively) than that of cisplatin (17.4) (Table 1). We observed that very limited cell death is evident, for both compounds, at 24 h, this rendering a classical proteomic approach well feasible.[28]

To analyze in detail protein expression modifications induced by the two drugs, proteomic analyses of A2780/R treated and untreated cells were performed. Representative 2D Coomassie-stained gels for control (panel C), *Auoxo6*-treated (panel A) and *Auranofin*-treated (panel B) A2780/R cells are shown in Fig. 2. An average of about 1300 protein spots was separated on each gel. To obtain statistically significant results, each sample was run in triplicate. Remarkably, both *Auranofin* and *Auoxo6* treatments caused small modifications of protein expression profiles. Only a limited number of protein spots manifested appreciable down- or up-regulation. When gels corresponding to untreated cells were used as a reference in gel analysis, the density of 38 spots was found to change significantly after both treatments. Spots with at least a 1.5-fold ($P < 0.05$) change in their expression level were considered as "significantly changed". Comparative analysis revealed that 18 spots were differentially expressed in cells treated with *Auranofin*; of these, 7 spots resulted to be up-regulated and 11 down-regulated. We found 20 spots differentially expressed in *Auoxo6*-treated cells: 7 up and 13 down-expressed. The locations of these protein spots are marked with circles in the representative gel shown in Fig. 2. Among these 38 differences excised from preparative Coomassie-stained 2-DE gels, in gel digested with trypsin and analyzed by mass spectrometry, we were able to identify 30 proteins. Not all spots could be identified because of the relatively low protein concentrations and MS sensitivity limitations. Positions of the identified spots are indicated by circles and numbers in the representative gels shown in Fig. 2. A list of the up- and down-regulated proteins is given in Table 2. The table reports all the identified proteins, their relative amounts including protein name, NCBI database accession number, Mascot score, peptide matched, sequence coverage and statistical analysis (fold change ≥ 1.5 and p-value < 0.05). A group of 10 protein spots (6, 7, 13, 8, 16, 11, 14, 17, 19 and 20) shows a significant down-expression in cells treated with *Auranofin versus* controls. A protein spot (15) shows an increase of the intensity level with a p-value of 0.037. The volume of 12 protein spots (19, 23, 25, 37, 26, 27, 32, 29, 33, 36, 38 and 34) was significantly down-regulated in the *Auoxo6*-treated cells with a p-value ranging between 0.05 and 0.005 when compared with the control group. Four protein spots (31, 24, 22 and 35) showed a significant volume increase in *Auoxo6*-treated cells.

Among the identified protein spots we found as down-expressed in both treatments the Thioredoxin-like protein 1 (TXL-1) (spot 19), involved in the regulation of cell redox homeostasis. In *Auranofin*-treated cells this protein resulted to

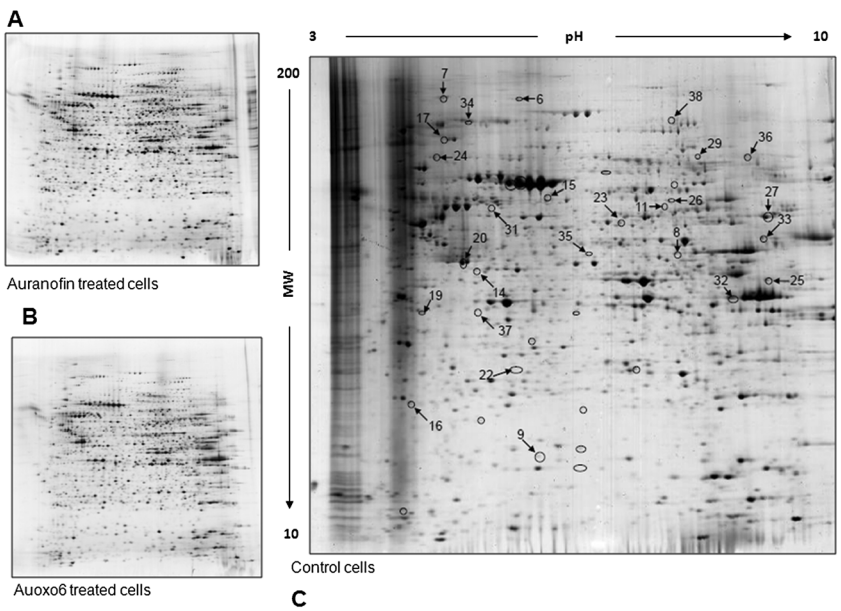

Fig. 2 Representative 2D gel images for: (A) A2780 *Auranofin*-treated cells, (B) A2780 *Auoxo6*-treated cells and (C) A2780 control cells. Circles indicate differentially expressed spots. Numbers indicate proteins identified by MS (see Table 2).

Table 2 Relative protein expression changes of *Auranofin*-treated cells *vs.* control cells and *Auoxo6*-treated cells *vs.* control cells. ND: Not Detected. Bold numbers indicate the spot changed after both treatments

Spot no.	Protein name	AC[a]	Score[b]	No. of matching peptides[c]	Sequence coverage[d] (%)	%V ($\times 10^{-4}$) mean ± (SD)[e]		Fold change[f]	
						Control	Treatment	Control/ treatment	P value
Spots decreased following *Auranofin* treatment									
6	Phosphoribosylformylglycinamidine synthase	O15067	118	14	13	502 ± 122	275 ± 119	1.83	<0.01
7	Hypoxia up-regulated protein 1	Q9Y4L1	134	11	15	457 ± 81	291 ± 109	1.57	<0.05
13	Proteasome subunit alpha type-6	P60900	119	8	33	428 ± 178	240 ± 96	1.79	<0.05
8	26S protease regulatory subunit 8	P62195	147	13	36	775 ± 210	518 ± 67	1.50	<0.05
16	Proteasome subunit beta type-6	P28072	119	7	32	671 ± 210	367 ± 231	1.83	<0.05
11	Nucleoporin p54	Q7Z3B4	104	8	25	243 ± 54	163 ± 56	1.49	<0.05
14	DNA-directed RNA polymerases I and III subunit RPAC1	O15160	101	7	28	631 ± 278	342 ± 81	1.84	<0.05
17	Transitional endoplasmic reticulum ATPase	P55072	182	19	31	253 ± 98	162 ± 23	1.56	<0.05
20	Actin, cytoplasmic 1	P60709	109	10	34	8031 ± 3922	3984 ± 2006	2.02	<0.05
19	Thioredoxin-like protein 1	O43396	153	11	54	503 ± 80	142 ± 99	3.52	<0.001
Spots increased following *Auranofin* treatment									
15	NADP-dependent malic enzyme + T-complex protein 1 subunit alpha	P48163 + P17987	83 + 73	9 + 9	23 + 17	164 ± 61	319 ± 146	0.51	<0.05
Spots decreased following *Auoxo6* treatment									
23	Aldehyde dehydrogenase X mitochondrial + RuvB-like 1	P30837 + Q9Y265	158 + 91	14 + 9	33 + 28	363 ± 29	207 ± 77	1.75	<0.005
25	DnaJ homolog subfamily B member 1	P25685	185	16	43	813 ± 161	531 ± 135	1.53	<0.01
37	Keratin, type I cytoskeletal 9	P35527	81	8	21	308 ± 116	187 ± 43	1.65	<0.05
26	Keratin, type II cytoskeletal 6B	P04259	100	8	17	160 ± 92	ND	ND	<0.005
27	Plasminogen activator inhibitor 1 RNA-binding protein	Q8NC51	124	14	29	3461 ± 426	2283 ± 901	1.52	<0.05
32	Glyceraldehyde-3-phosphate dehydrogenase	P04406	115	10	32	2610 ± 729	1531 ± 763	1.70	<0.05
29	Far upstream element-binding protein 2	Q92945	150	13	25	179 ± 46	114 ± 35	1.57	<0.05
33	Splicing factor 3B subunit 4	Q15427	109	9	31	784 ± 248	480 ± 156	1.63	<0.05
36	Splicing factor 1	Q15637	92	8	14	531 ± 147	274 ± 114	1.93	<0.01
38	Elongation factor 2	P13639	113	14	19	265 ± 46	158 ± 99	1.67	<0.05
34	Heat shock protein 105 kDa + actin, cytoplasmic 2	Q92598 + P63261	157 + 88	18+9	26 + 29	345 ± 116	184 ± 47	1.88	<0.05
19	Thioredoxin-like protein 1	O43396	153	11	54	503 ± 80	276 ± 28	1.82	<0.05
Spots increased following *Auoxo6* treatment									
31	T-complex protein 1 subunit theta	P50990	110	8	22	123 ± 32	220 ± 36	0.56	<0.01
24	Tubulin alpha-1A chain o 1B	Q71U36 + P68363	86	9	35	358 ± 69	564 ± 126	0.64	<0.05
22	Serine/arginine-rich splicing factor 9	Q13242	117	9	36	27 ± 78	255 ± 78	0.10	<0.005
35	Ornithine aminotransferase. Mitochondrial	P04181	104	7	24	90 ± 20	228 ± 69	0.39	<0.005

[a] Swiss-Prot/TrEMBL accession number. [b] MASCOTscore (Matrix Science, London, UK; http://www.matrixscience.com). [c] Number of peptide masses matching the top hit from Ms-Fit PMF. [d] Percentage of amino acid sequence coverage of matched peptides in the identified proteins. [e] Each value represents the mean and SD of individually computed %V (V = integration of OD over the spot area; %V = V single spot/V total spots) in three different gels of control, *Auranofin* and *Auoxo6*. [f] Fold change (control *vs.* *Auranofin* and control *vs.* *Auoxo6*) was calculated dividing %V from control by the %V from *Auranofin* and from *Auoxo6*.

be reduced in expression with a p-value of <0.005; in *Auoxo6*-treated cells the corresponding p-value was <0.05. Fig. 3 shows the relative amounts and statistical analysis for this protein in *Auranofin*-treated and *Auoxo6*-treated cells *versus* control cells. In Fig. 3 are also shown magnified regions from triplicate 2D gel images with this spot.

Altered proteins upon Auranofin treatment

Among proteins identified as down-regulated after treatment with *Auranofin* we found phosphoribosylformylglycinamidine synthase, a protein involved in purine metabolism (spot 6), Hypoxia up-regulated protein 1 (HYOU1) (spot 7), that belongs to the heat shock protein 70 family and has a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation. This protein may play a role as a molecular chaperone and participate in protein folding. We have noticed a down expression of the 26S protease regulatory subunit 8 (spot 8), a protein involved in the ATP-dependent degradation of ubiquitinated proteins. Two proteins with an ATP-dependent proteolytic activity: the proteasome subunit alpha (spot 13) and beta (spot 16) type-6, belonging to a multi-catalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu and Glu adjacent to the leaving group. The subunit beta is responsible for the peptidyl glutamyl-like activity.

Moreover resulted down expressed: the RNA polymerases I and III subunit RPAC1(spot 14) which catalyzes the

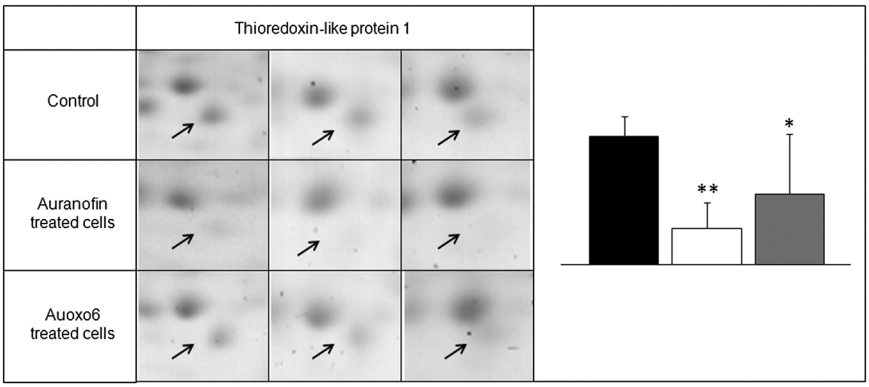

Fig. 3 Magnified regions of 2D gel images indicating spot 19, corresponding to Thioredoxin-like protein 1. In the column on the right the relative histogram is reported. Black bars, white bars and gray bars represent control, *Auranofin* and *Auoxo6* treatment, respectively. Bars represent the mean ± the standard deviation of spot volume percentage from three different experiments. The volume percentage is calculated as V single spot/V total spots (V is the integration of the optical density over the spot area). Asterisks indicate statistically significant differences with $P < 0.05$ (*) and $P < 0.001$ (**).

transcription of DNA into RNA; the cytoplasmic Actin (spot 20), involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells, the Nucleoporin p54 (spot 11), a component of the nuclear pore complex, required for the trafficking across the nuclear membrane and Transitional endoplasmic reticulum ATPase (TERA) (spot 17), involved in protein folding.

In one spot (spot 15) showing a significant positive correlation with *Auranofin* treatment we found two proteins: the NADP-dependent malic enzyme, that generates NADPH for fatty acid biosynthesis and a molecular chaperone, the T-complex protein 1 subunit alpha. The activity of the first enzyme links the glycolytic and citric acid cycles; the second protein assists the folding of proteins upon ATP hydrolysis. Moreover, this protein is known to play a role, *in vitro*, in folding of actin and tubulin.

Altered proteins upon *Auoxo6* treatment

Fifteen protein spots were significantly correlated with *Auoxo6* treatment; these proteins are listed in Table 2. Eleven spots showed negative correlation while four spots showed positive correlation with treatment. Significant negative correlations were found concerning spot 23, identified as the mitochondrial aldehyde dehydrogenase X, with traces of RuvB-like 1 protein. This mitochondrial protein plays a major role in the detoxification of alcohol-derived acetaldehyde. The second protein is involved in transcriptional activation of selected genes by nucleosomal histone acetylation. This complex seems to be required for the activation of transcriptional programs associated with oncogenes and proto-oncogenes. We found, as down-expressed, a protein that interacts with HSP70 and can stimulate its ATPase activity: this protein is the DnaJ homolog subfamily B member 1 (spot 25). Two cytoskeletal Keratins, type I (spot 37) and type II (spot 26), were also observed to be down expressed.

Two proteins related to mRNA resulted as down-expressed: the plasminogen activator inhibitor 1 RNA-binding protein (spot 27) and the far upstream element-binding protein 2 (spot 29). These two proteins play a role in the regulation of mRNA stability and in mRNA trafficking respectively. Other proteins showing the same trend are two splicing factors: splicing factor 3B subunit 4 (spot 33) and splicing factor 1 (spot 36). Also, an elongation factor 2 (spot 38), a heat shock protein 105 kDa and the cytoplasmic Actin 2 (both in the same spot 34) revealed a similar trend. The glyceraldehyde-3-phosphate dehydrogenase (spot 32) playing a role in glycolysis and nuclear functions resulted to be reduced in expression after treatment. It participates in nuclear events including transcription, RNA transport, DNA replication and apoptosis.

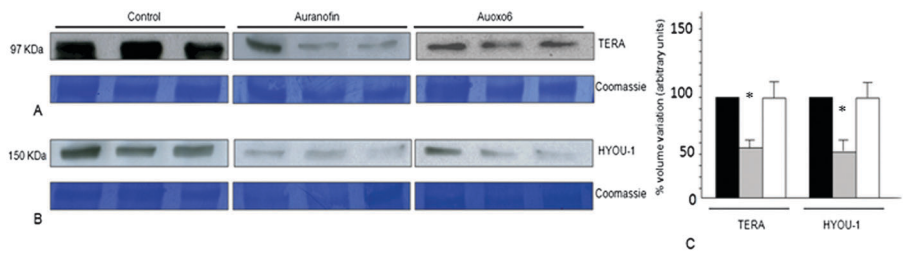

Fig. 4 Validation of proteomic results by western blot analysis. Western blot was probed with antibodies against TERA and HYOU1, proteins identified by proteomic screening. The intensity of immunostained bands was normalized with the total protein intensities measured from the same blot stained with Coomassie brilliant blue (in panel A and panel B a representative band of the lane is reported). *Auranofin* treatment induced a decrease of expression of TERA (A) and HYOU1 (B) after 24 h. (C) Histograms representing TERA and HYOU1 protein expression variation. Bars represent the mean ± the standard deviation of western blot signals from three different experiments, expressed as arbitrary units. Black bars represent control cells, grey bars represent Auranofin-treated cells and white bars represent Auoxo6-treated cells. Asterisk indicates that the difference is statistically significant, $P < 0.05$. The two-tailed non-paired Student's t-test was performed using ORIGIN 6.0 ($p < 0.05$).

Among proteins whose expression showed a positive correlation with *Auoxo6* treatment, we identified a molecular chaperone which assists protein folding, the T-complex protein 1 subunit theta (spot 31); the major constituent of microtubules, protein tubulin alpha-1A chain (spot 24), a protein involved in splicing, the serine/arginine-rich splicing factor 9 (spot 22) and a mitochondrial protein involved in amino-acid biosynthesis, ornithine aminotransferase (spot 35).

To validate the obtained results, as well as to further evaluate the nature and importance of some of the identified proteins that changed expression after drug treatment, monodimensional (1D) western blotting analyses were performed. For these analyses, a new experiment was performed on A2780/R cells either untreated or treated for 24 h with *Auranofin* and *Auoxo6*. Two proteins, TERA (spot 17) and HYOU1 (spot 7), were validated by western blotting. As shown in Fig. 4, TERA and HYOU1 are both down-expressed in *Auranofin*-treated cells. The protein expression-fold changes are consistent with the reported 2-D results.

Discussion

Our previous proteomic study reporting the effects of *Auranofin* and *Auoxo6* on A2780 cisplatin-sensitive cells[21] indicated that *Auranofin* and *Auoxo6* have a similar mode of action. The protein pattern modifications induced by both gold compounds on those cells were limited; notably, some of the affected proteins were in common and were involved in redox homeostasis and induction of apoptosis. In the present paper we extended that kind of investigation to the parent cell line resistant to cisplatin (A2780/R) to highlight the mechanisms of action of these two representative gold compounds in overcoming platinum-based drug resistance. As reported in Table 1, *Auranofin* and *Auoxo6* manifest different cytotoxic properties *versus* A2780/S and A2780/R cells. In particular, *Auranofin* shows roughly the same cytotoxic activity in both cell lines ($r = 0.94$), while *Auoxo6* is more active in sensitive cells compared to resistant ones ($r = 2.4$). This different behaviour could be somehow related to the different proteomic profiles observed in our experiments. It is noteworthy that both treatments, though causing extensive cell death at 72 h, induce very limited proteome changes at 24 h. The affected proteins that were later identified participate in a variety of cellular processes, such as cell structural organization, defence against oxidative stress, transcription and protein degradation. Interestingly, we found that Auranofin reduced the amount of HYOU1, a molecular chaperone involved in protein folding. This protein has a protective role in cells because it contributes to apoptosis suppression under hypoxic conditions.[29] Miyagi *et al.* showed that tumorigenicity is reduced in prostate cancer by decreasing HYOU1 expression.[30] Namba *et al.* reported that up-regulation of HYOU1 in cancer cells can inhibit apoptosis.[31] We may assume that HYOU1 correlates with chemotherapeutic resistance and that gold compounds could overcome drug resistance by decreasing expression levels of apoptotic suppressors. We also found that Auranofin decreases the expression of proteasome subunits. These proteins are responsible for protein degradation. The ubiquitin/proteasome pathway is indeed the primary system for extralysosomal protein degradation, necessary for maintaining normal cellular functions. In recent years, inhibitors of the proteasome were suggested as novel antitumor agents in cancer therapy.[32] Tumor cells are in fact more susceptible than normal cells to these inhibitors because of the burden placed on the proteasome due to the increased extent of damaged proteins under stress conditions.[33] Notably, it was previously reported by many authors that proteasome inhibitors trigger apoptosis. Moreover, Auranofin treatment, under our experimental conditions, reduces the amount of TERA, a protein necessary for the export of misfolded proteins from endoplasmic reticulum. Overall, modification of the protein degradation pathway and the inhibition of the expression of the proteasomal ex apparatus could be associated with apoptotic cancer cell death induced by this drug. As far as the effects of Auoxo6 are concerned, we discovered a reduced expression in proteins related to mRNA transcription, stability, trafficking and splicing. Among them far upstream element-binding protein 2 (FUBP2), which is known to be over-expressed in tumor cells together with FUBP1,[34] plays a role in mRNA trafficking and splicing. This over expression supports tumor cells proliferation and migration. In contrast, its reduced expression level, found in our experiments, could be responsible for the cytotoxic effects of Auoxo6 on A2780 cells. We also found a reduced expression of cytoskeletal proteins. Multiple evidence suggests that cells use cytoskeleton dynamic state as an indicator of cellular health. Many authors showed that reorganization of cytoskeletal proteins correlates with apoptosis induction[35,36] and it is known that ROS are involved in remodeling the actin cytoskeleton.[37,38] Among the most significant proteins involved in cytoskeletal reorganization we identified cytoplasmic Actin and two types of Keratins.[39] We found a down-regulation of two isoforms of Actin: actin 1 or beta-actin after *Auranofin* treatment and actin 2 or gamma-actin after *Auoxo6* treatment, suggesting that these gold compounds may act by a mechanism involving remodeling of the cytoskeleton and alteration of the cell shape, which could lead to an apoptotic death. Furthermore, we found a protein whose expression level is strongly reduced after both treatments: this protein is TXL-1; it is involved in cell redox homeostasis.[40] Oxidative stress is characterized by the depletion of the general antioxidant systems leading to an alteration of the cellular redox status. Thus, the balance between reactive oxygen species production and antioxidants determines the degree of oxidative stress.[41] Thioredoxins have emerged as an essential family of proteins directly related to the antioxidant cellular network.[42] We observed a significant two-fold down expression of this protein after both treatments; this fact could be related to the cytotoxicity of these gold compounds. We know that cancer cells exist in a stressed environment and rely on the Trxs for protection against stress-disregulated redox signaling. By triggering a reduction in expression of a protein with important antioxidant functions gold compounds could determine massive oxidative stress that eventually leads to cellular death.

Moreover the proteomic approach could be useful to understand the resistance mechanisms involved in platinum resistance as reported by Yan *et al.*[43] They identified five proteins to become candidates for platinum resistance and useful for further study of screening of resistant biomarkers.

Conclusions

Proteomics methods have the potential to provide specific insight into the alterations induced by drugs on protein expression. In turn, the observed proteomic alterations may be related to the modes of action of the drugs themselves. We have used such an approach to investigate the molecular mechanisms through which two cytotoxic gold based drugs *i.e. Auranofin* and *Auoxo6* cause their biological effects. Notably, highly different proteomic alterations were detected for the two investigated metallodrugs suggesting a substantially different mechanism of action. It is found that *Auranofin* mostly acts by altering the amount of proteasome proteins while *Auoxo6* mainly modifies proteins related to mRNA splicing, trafficking and stability. However, we also observed that a protein involved in oxidative stress defence, *i.e.* Thioredoxin-like protein 1, is greatly reduced after treatment with both gold compounds. Our proteomic results suggest the putative targets of these compounds. Extending the analysis to the transcription level will better explain if the observed differences in protein amounts are caused by transcriptional or post-transcriptional events. Overall, these findings may contribute to elucidate the molecular mechanisms of the tested drugs and offer insight into their respective modes and sites of biological action.

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